CHARACTERIZATION OF A 1,2-PROPANEDIOL PRODUCING *ESCHERICHIA* STRAIN ISOLATED FROM A GEOTHERMALLY HEATED INTERTIDAL POOL IN NORTHERN ICELAND

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KEYWORDS

- Deoxy Sugars
- Culture Conditions
- Propylene Glycol
- *Enterobacteriaceae*

ABSTRACT

The ability of a mesophilic isolate, strain CC5C, isolated from a temperate geothermally-heated intertidal pool in northern Iceland is described herein. Strain CC5C belongs to the family *Enterobacteriaceae* with greater than 99.1% similarity to *Escherichia marmotae* and *Shigella dysenteriae* based upon 16S rRNA gene analysis. Strain CC5C is a facultative anaerobe exhibiting growth between 5 and 50°C with an optimal growth at 40°C, initial pH values between 4.0 and 9.0 with an optimum at pH 7.5. Strain CC5C, unlike its nearest phylogenetic neighbors, degrades starch, dulcitol, and sucrose as well as potentially cellobiose which are uncommon features among these genera. Under aerobic conditions the catabolism of L-rhamnose and L-fucose revealed that the dominant product was 1,2-propanediol while under anaerobic conditions it was a mixture of acetate and 1,2-propanediol. The effect of increased initial substrate concentration was investigated for glucose, L-fucose, and L-rhamnose with inhibition apparent at concentrations above 20 mM under both anaerobic and aerobic conditions. Although, differences in end product formation were observed between aerobic and anaerobic conditions for L-rhamnose. Strain CC5C rapidly catabolizes rhamnose and produces 1,2-PD at a rate of 3.41 mmol/h on L-rhamnose and 2.37 mmol/h on a mixture of glucose and rhamnose with up to 96% of the theoretical yield on L-rhamnose.

INTRODUCTION

Knowledge regarding the production of 1,2-propanediol (1,2-PD) by bacteria from methylpentoses has been widely limited to clinical isolates and soil bacteria with work on organisms from extreme environments as well as the marine ecosystems being lacking. Geothermally-heated intertidal pools represent unique environments that are an interface between the marine and freshwater environments. The tides bring a regular influx of nutrients, with some serving as carbon sources for multiple microbe species. Organic material deposited in intertidal pools can include terrestrial and marine materials,

which provide a source of protein, lipids, and carbohydrates to microbes existing within the pool. Macro algae often contain methylpentoses not commonly found in terrestrial biomass; the methylpentoses L-fucose and L-rhamnose composition of a given macro algae species is highly dependent upon the type of macro algae. For example, green algae within the genus of *Ulva* contain ulvan, which is composed of L-rhamnose, while brown algae species, including *Laminaria*, *Fucus*, and *Ascophyllum* species, contain fucoidan, which is comprised on L-fucose. Given the influx of macro algae into geothermally-heated intertidal niches, this is a logical environmental niche in which to look for organisms degrading macro algae components.

1,2-PD (α-propylene glycol) is a chiral three-carbon diol with two enantiomers, *(R)*-1,2-PD and *(S)*-1,2-PD, although it is commonly sold commercially as a racemic mixture. In its pure form, 1,2-PD is clear, colorless, and highly viscous which is also odor- and tasteless. 1,2-PD is generally regarded as benign and has been accepted as safe status (GRAS). 1,2-PD has many applications and is used in antifreeze products, disinfectants, lubricants, artificial smoke, as a solvent, or as a moisturizer in cosmetics, plastics, paint, pharmaceuticals, and food products (10, 24). Commercially available *rac*-1,2-PD is produced by the hydrolysis of propylene oxide which is a product of petroleum reformation, while enantiomerically pure *(S)*-1,2-PD can be produced microbially from L-fucose and L-rhamnose and enantiomerically pure *(R)*-1,2-PD can be produced from glucose *via* the methylglyoxal bypass (24).

During methylpentose metabolism, L-rhamnose and L-fucose are converted to their corresponding phosphate intermediates, fuculose-1-phosphate and rhamnulose-1-phosphate, by inducible parallel pathways each including a permease, isomerase, kinase and aldolase, which are then converted to dihydroxyacetone (DHAP) and L-lactaldehyde. Under anaerobic conditions, L-lactaldehyde is reduced to *(S)*-1,2-PD *via* the action of propanediol oxidoreductase. Under aerobic conditions, L-lactaldehyde is oxidized to L-lactate *via* lactaldehyde dehydrogenase and then further to pyruvate. The methylglyoxal pathway is an offshoot branch of glycolysis where DHAP is converted to methylglyoxal and then D-lactaldehyde. The end-products, depending on oxygen conditions, are either *(R)*-1,2-PD or pyruvate (2, 3).

The catabolism of sugars leading to 1,2-PD production has been examined in several organisms of which the methylpentose metabolism of *Escherichia coli* strain K-12 was extensively studied (4, 5). Under aerobic conditions, active lactaldehyde dehydrogenase is induced in both fucose- and rhamnose-grown cells and a basal level expression of inactive propanediol oxidoreductase is observed in fucose-grown cells. This allows for much quicker adaptation of fucose-grown cells to anaerobiosis, although final enzymatic activities are the same. The propanediol oxidoreductases produced by L-rhamnose-grown cells and fucose-grown cells are immunologically identical, which strongly suggests that they are products of a single gene located in the fucose locus (*fucO*) and when disrupted, L-rhamnose (as well as L-fucose) fails to induce propanediol oxidoreductase (11).

The role of phosphate limitation in the regulation of the methylglyoxal pathway and subsequent production of *(R)*–1,2–PD has as well been detailed for *Clostridium sphenoides* as described by (31). Conversion of sugars, including glucose and xylose to 1,2–PD has also been reported by *Salmonella typhimurium* under aerobic conditions (5) and by the strictly anaerobic thermophilic mutant of *Thermoanerobacterium thermosaccharolyticum* strain HG–8 (2, 8). More recently, mention of the ability of extremely thermophilic *Caldicellulosiruptor* species to catabolize L–rhamnose to 1,2–PD has also been described (6) and found to be a common feature in the genus (14).

The aim of this study was to examine *Escherichia* strain CC5C isolated from geothermally–heated intertidal pools north of Húsavík, north central Iceland, which preliminary screening identified as a 1,2–PD producing organism from methylpentoses. Since the ecosystem of the these intertidal pools consists largely of methylpentose–rich macro algae, serving as a renewable carbon source, it was hypothesized that the microbes within that ecosystem would be able to ferment common sugars to 1,2–PD while demonstrating a broader tolerance to environmental stresses due to the highly variable geophysical conditions associated with both geothermally heated fresh water and the tidal influx of saline water.

MATERIALS AND METHODS

MATERIALS

All reagents were acquired from Sigma–Aldrich unless stated otherwise. Brilliant Green Agar (BGA), Eosin–Methylene Blue Agar (EMB), MacConkey Agar (MAC), Mannitol Salt Agar (MSA), Yeast extract (YE), Reasoner's 2A Agar (R2A), and Plate Count Agar (PCA) were obtained from Difco. L–fucose and fucoidan were obtained from Dextra (Reading, UK). Azure–linked β–glucan and xylan were obtained from Megazyme. Nitrogen used for headspace gas contained less than 5 ppm of oxygen. *E. coli* (DSM 30083) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany).

CULTIVATION MEDIUM

Anaerobic cultivations were performed in Basal Mineral Medium (BM) as previously described by (21). The culture medium consisted of (per liter): NaH_2PO_4 2.34 g, Na_2HPO_4 3.33 g, NH_4Cl 2.2 g, NaCl 3.0 g, $CaCl_2$ 8.8 g, $MgCl_2$ x 6H2O 0.8 g, yeast extract 2.0 g, resazurin 1 mg, trace element solution 1 mL, vitamin solution 1 mL and $NaHCO_3$ 0.8 g. Carbon and energy sources were 20 mM or in the case of polymeric

substrates, 2 g L^{-1}. The trace element solution consisted of (per liter): $FeCl_2$ x $4H_2O$ 2.0 g, EDTA 0.5 g, $CuCl_2$ 0.03 g, H_3BO_3, $ZnCl_2$, $MnCl_2$ x $4H_2O$, $(NH_4)_6Mo_7O_{24}$, $AlCl_3$, $CoCl_2$ x $6H_2O$, $NiCl_2$, all 0.05 g, and 1 mL of concentrated HCl. The medium was reduced with a solution of Na_2S x $9H_2O$, 0.3 g. The vitamin solution was prepared according to DSM141. Media was autoclaved for 60 minutes after which vitamins, trace elements, and carbon sources were added through a syringe filter (Whatman, PES 0.22 µm) from sterile stock bottles. All experiments were performed at 35°C at pH 7.0 with a liquid–gas phase ratio of 1:1 without agitation unless otherwise noted. Aerobic cultivations were performed using BM medium with the omission of sodium sulphide and cysteine solution. The inoculum volume was 2% (v/v); anaerobic cultures were cultivated without shaking while those grown aerobically were shaken at 125 rpm unless otherwise noted. All experiments were performed in triplicate.

2.2 ISOLATION OF STRAIN CC5C

A geothermal fluid sample from a geothermally heated intertidal pool (66° 03.451 N, 17° 21.466 W, 40.3°C, 5.7 mS) was collected with a sampling pool in a sterile 50 mL polypropylene sample tube in an area north of Husavik (north central), Iceland in June, 2015. The sample was enriched using a 2% (v/v) inoculation volume in Basal Mineral Medium (BM) that contained 0.2% (v/v) rapeseed oil and incubated at 40°C under aerobic conditions without shaking. The culture was subcultured three times prior to streaking on PCA. A single isolated colony was selected and repurified two additional times by streaking on PCA. The culture was maintained on PCA and cryopreserved at –20°C in 30% (v/v) glycerol.

2.3 STRAIN CHARACTERIZATION AND SUBSTRATE SPECTRA

Strain CC5C was evaluated using API 20E, API 20NE, API 50CH, and API ZYM test strips (BioMerieux, France) according to manufacturer's directions using *E. coli* DSM 30083 as a control. Voges–Proskauer, 3% KOH string test, and peroxidase test were performed using standard methods (30). Oxidase activity was evaluated using test discs (Sigma–Aldrich) according to manufacturer's directions. Gram staining was performed according to (30). Oxidative Fermentation (OF) tests were conducted according to (30). Characteristics on selective and differential agars (30) were performed in accordance with the manufacturer's directions.

The utilization of starch, casein, and cellulose was evaluated on PCA plates containing 2% (w/v) of the target substrate and visualized according to (30). Alginate utilization was evaluated on R2A plates prepared with the inclusion of 2% (w/v) sodium alginate with the exclusion of starch and glucose; visualization was performed using Gram's Iodine solution. Plate-based inorganic phosphate solubilization was evaluated using NBRIP medium according to the method of Nautiyal (20). Chitinase degradation was evaluated using the method of (13) except with the omission of agar for the detection of

chitin degradation in liquid medium. Azure-linked β-glucan and xylan (0.2% w/v) was used to evaluate the degradation of β-glucan and xylan by supplementing Reasoner's 2A agar.

The temperature range was determined qualitatively by incubating streaked PCA plates at temperatures ranging from 5 to 60°C in 5°C increments. Growth curves were generated by performing kinetic experiments in triplicate using tryptic soy broth (Difco, TSB), in microtiter plate from a stock culture in the exponential growth phase; a 2% (v/v) inoculation volume was used in all cases. The increase in OD was measured using a Bioscreen C (GrowthCurves Ltd, Finland) as independent triplicates at each temperature from 20 to 65°C in 5°C increments. Maximum specific growth rate (μ_{max}) and generation time (g) were calculated according to (1).

pH and sodium chloride growth range and optima were examined in TSB containing 50 mM phosphate buffer (from pH 4 to 9 in 0.5 unit increments) and sodium chloride (from 0 to 9% w/v) at the T_{opt} of the strain in the manner described above.

2.2 FERMENTATION OF SINGLE SUBSTRATES

Hexoses, pentoses, methylpentoses, sugar alcohols, and polyalcohols were used at a concentration of 20 mM while disaccharides were used at a concentration of 10 mM. All polymeric substrates were used at a concentration of 0.2% (w/v). Experiments were performed anaerobically in serum bottles at pH 7 with liquid-gas phase ratio of 1:1 for all experiments.

2.3 EFFECT OF INITIAL SUBSTRATE CONCENTRATION

The effect of initial substrate concentration was evaluated for three substrates (glucose, L-rhamnose, and L-fucose) in batch cultures from 10 to 60 mM. The cultures were incubated at 35°C for five days under both aerobic and anaerobic conditions. After incubation the end products were analyzed.

2.4 KINETIC EXPERIMENTS

Kinetic experiments were performed both aerobically and anaerobically in 125 mL Erlenmeyer flasks or serum bottles without agitation. Substrates were used at a concentration of 20 mM glucose, L-rhamnose, L-fucose, *rac*-1,2-PD, and control. Fermentation broths were sampled (1 mL) and analysed for optical density over a period of 46 hours; withdrawn samples were stored at -20°C for further analysis.

2.5 ANALYTICAL METHODS

Optical density was determined using a Shimadzu UV-1800 UV-Visible spectrophotometer at a wavelength of 600 nm and a pathlength of 1 cm. Cell-free solutions were prepared by centrifugation at 13000 g for 5 min. Volatile end products, such as ethanol and volatile fatty acids were measured using a Clarus 580 (Perkin Elmer) gas chromatograph equipped with a FID as described by Orlygsson & Baldursson (18); samples were prepared by mixing 100 µL

formic acid (25% w/v), 100 µL crotonic acid (0.1% w/v), and 600 µL dH_2O with 200 µL of cell-free sample. Hydrogen was analysed using a AutoSystem XL (Perkin Elmer) gas chromatograph equipped with a TCD using the method of (21). Glucose concentration was determined colorimetrically using the anthrone method described (18) with minor modifications; 50 µL cell-free sample was mixed with 250 µL of 0.1% (w/v) anthrone in concentrated sulphuric acid and incubated for 20 minutes at 100°C, cooled to room temperature and then read on a microplate reader (Bioscreen C, Growth Curves Ltd, Finland) at 600 nm against a water blank. Methylpentoses were analysed colorimetrically according to the method of (12). 1,2-propanediol was analysed using the colorimetric method of Jones (15) modified for use in microplates; 250 µL of concentrated sulphuric acid was added with 50 µL of cell-free sample in a microplate which was then incubated at 70°C for 20 min; after cooling to room temperature, 10 µL of a 3 % w/v ninhydrin solution in 5% w/v sodium bisulfite was added and the plate was mixed (200 rpm, 60 sec) and allowed to develop for 1 hr in the absence of light and then read on a microplate reader (Bioscreen C) at a wavelength of 600 nm. Lactate was quantified colorimetrically according to the method of Taylor (28) with the following modifications: 300 µL of concentrated sulphuric acid was added with 50 µL of sample in an Eppendorf tube. The sample was incubated in a boiling water bath (~100°C) for 10 minutes and then cooled to room temperature, after cooling 5 µL of 4% (w/v) copper(II) sulphate and 10 µL of 1.5% (w/v) *p*-phenyl phenol (in 95% v/v ethanol) was added. The sample was vortexed and incubated at room temperature for 30 minutes. Samples were read at 580 nm against a water blank.

2.6 MOLECULAR METHODS

The 16S rRNA gene from *Escherichia* strain CC5C was sequenced (1507 nt). The sequence was originally compared to sequences in the NCBI (National Center for Biotechnology Information) database using the nucleotide BLAST. The top sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using a global alignment algorithm which was implemented at the EzTaxon server (23). The most similar sequences obtained were aligned with sequencing results in MEGA6 (27) and the maximum likelihood method based on the Tamura-Nei model was used to generate a phylogenetic tree (26). Genomic DNA extraction was carried out, by using UltraClean™ Microbial DNA Isolation Kit from MoBio Laboratories, according to the manufacturer's protocol. PCR mediated amplification of the 16S rDNA and purification of the PCR product was carried out with 519F (CAGCAGCCGCGGTAATAC) and 926R (CCGTCAATTCCTTTGAGTTT) universal primers as previously described (21). The amplified PCR products were purified with Exo-SAP reaction; Substrate preparation was prepared by mixing 0,025 µL of Exonuclease I (NEB, England), 0,05 µL of Antarctic Shrimp Phosphatase (NEB, England) and 9,925 µL of MQ water for each sample. 10 µL of the reagent substrate was

then mixed with 20 µL of amplified PCR products. The reaction was carried out with PTC-200 (MJ-Research) for 3 min at 95°C for the initial denaturation, 30 sec at 95°C for denaturation, 30 sec at 50°C for annealing, 90 sec at 68°C for extension, and a final extension time of 7 min at 68°C followed by storage at 4°C. Purified PCR products were then transferred into 96-well MicroAmp plate, with either 519F or 926R primer, since the products were sequenced from each end, and sent to Macrogen (Netherlands) for sequencing.

RESULTS

STRAIN ISOLATION AND 16S SEQUENCING

Strain CC5C was isolated from a geothermally-heated intertidal pool north of Husavik in north central Iceland. The strain produced creamy circular colonies that are off-white in color when grown on PCA and R2A. The strain was more than 99% similar to *Escherichia coli* based upon analysis of partial 16S rDNA (1507 nt) using nucleotide BLAST. Further analysis of the sequence with EZ Taxon indicates that CC5C is highly similar to *Escherichia marmotae* (99.13%), *Shigella dysenteriae* (99.13%), *Escherichia fergusonii* (99.06%), *Escherichia coli* (98.99%), *Shigella flexneri* (98.99%), and *Shigella sonnei* (98.85%), respectively. Preliminary screening of the strain revealed that the isolate CC5C was capable of producing 1,2-PD from L-rhamnose and was chosen for further investigation.

TRAIN CHARACTERIZATION AND SUBSTRATE SPECTRA

Strain CC5C is Gram-negative, string test positive, oxidase, and catalase positive. Strain CC5C is both oxidative and fermentative based upon positive oxidative fermentation tests. Colonies grown on PCA had an off-white circular morphology. Colonies of CC5C produced positive reaction on brilliant green agar, growth on MacConkey's with colonies presenting as pink, and produced purple colonies with a metallic green sheen on EMB agar plates characteristic of *E. coli*. No growth was observed on mannitol salt agar. Growth on starch impregnated plates followed by staining with Gram's iodine solution indicated starch being degraded.

CC5C has a growth range from 5 to 45°C with a temperature optimum of 40°C (Figure 1). Growth was observed between pH 4.0 and 9.0 with an optima of pH 7.5, and between sodium chloride concentrations between 0 and 7% w/v with optima of 0.5% (Figure 2). Maximum specific growth was 1.10 h^{-1} with a generation time of 37.8 min.

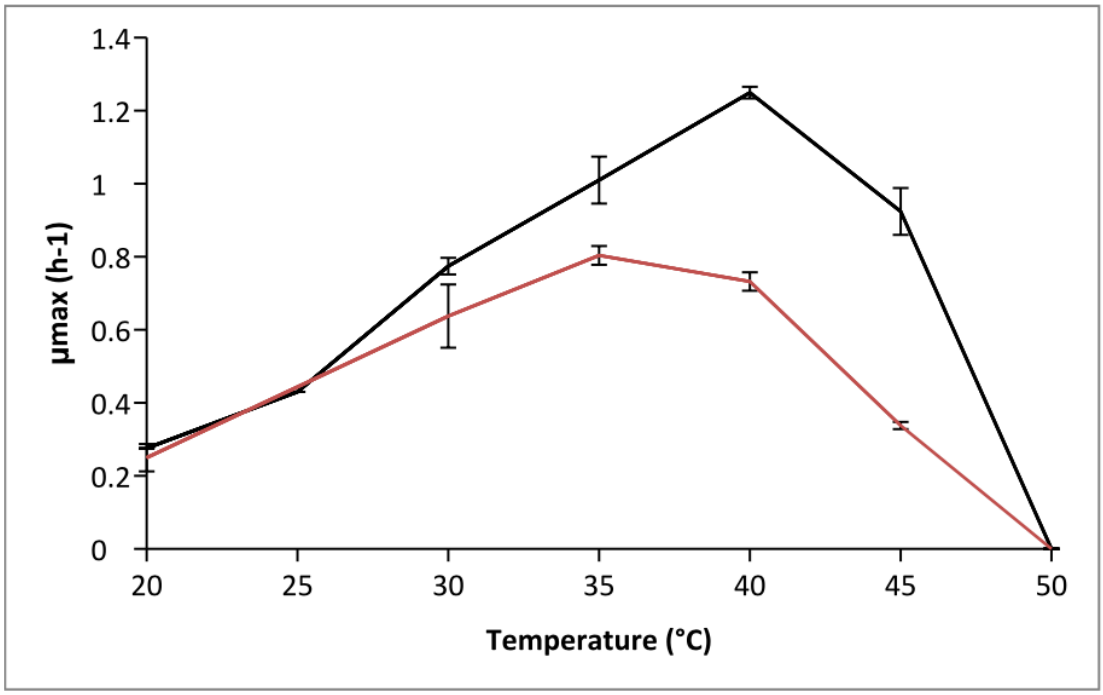

Figure 1 – Temperature optima of strain CC5C (black line) and *E. coli* (red line, DSM 30083) grown in TSB. Values represent average of triplicates with the standard deviation presented as error bars.

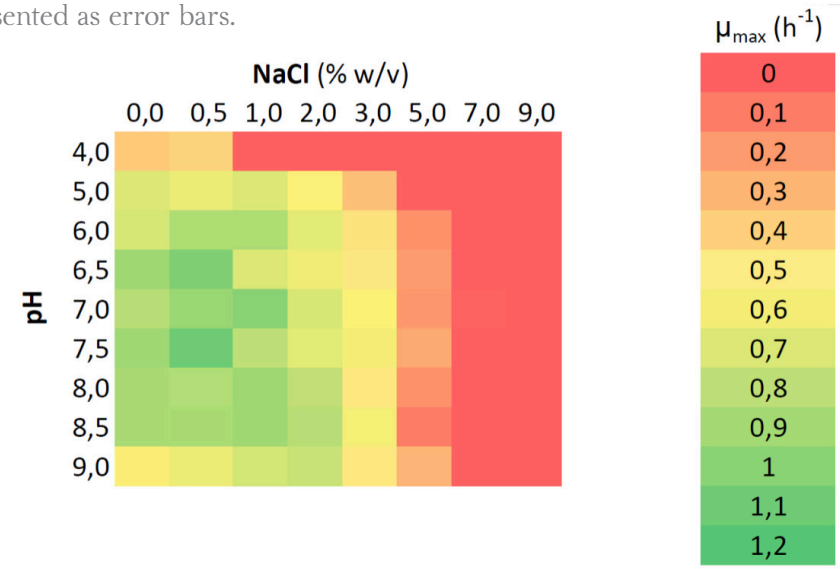

Figure 2 Optimization of pH and NaCl for maximum specific growth rate at Topt (40°C); values represent the average of triplicates (standard deviation not shown).

The substrate spectra of CC5C was evaluated using API test panels in parallel with the type strain of *E. coli* (DSM 30083); results for commonly used taxonomic features of closely related *Escherichia* and *Shigella* type strains are presented in Table 1.

	Strain								
Characteristic	**1**	**2**	**3**	**3A**	**4**	**5**	**6**	**7**	**8**
$T_{min/opt/max}$ (°C)	5/40/45	25/ND/37	ND/40/45[b]	ND/40/45	NR	NR	NR	NR/37/NR	NR/37/NR
$pH_{min/opt/max}$	4/7.5/9	ND/ND/ND	NR	4/7/9	NR	NR	NR	NR	NR
$NaCl_{min/opt/max}$	0/0.5/7	0/1/6	0/2/5[b]	0/0-0.5/5*	NR	NR	NR	NR	NR
Indole	+	-	+	+	-	+	-	d	-
ONPG[a]	+	-	+	+	+	+	+	d	+
Lysine decarboxylase	+	+	+	+	+	-	+	-	-
Ornithine decarboxylase	+	-	+	+	+	+	-	-	+
Fermentation of									
Starch	+	NR	-	-	NR	NR	NR	NR	NR
Sucrose	+	-	-	-	-	-	-	-	-
Dulcitol	+	-	-	-	-	d	-	-	-
Lactose	+	-	+	+	-	-	-	-	+
D-Mannitol	+	+	+	+	+	+	+	+	+
Adonitol	-	-	-	-	-	-	-	-	-
Raffinose	+	-	+	-	-	-	+	-	+
L-Rhamnose	+	+	+	+	-	+	+	d	+
D-Xylose	+	+	+	-	-	+	+	-	-
Melibiose	+	-	-	+	-	-	+	-	-
Cellobiose	-	-	-	-	-	+	+	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-
D-Sorbitol	+	+	+	+	-	-	-	d	-
Glycerol	w	+	-	w	-	-	-	-	d

Table 1 – Comparison of biochemical characteristics between strain CC5C and closely related species. Strains: 1, Strain CC5C (this study); 2, *Escherichia marmotae* HT073016T; 3, *Escherichia coli*; 3A, *Escherichia coli* (DSM 30083, this study), 4, *Escherichia* albertii LMG 20976T; 5, *Escherichia hermannii* CIP 103176T; 6, *Escherichia vulneris* ATCC 33821T; 7, *Shigella dysenteriae*; 8, *Shigella sonnei*. Data for *Escherichia* spp. from (19) and (17) and references therein, data for *Shigella* spp. is from (25). a ONPG, o-nitrophenyl-β-D-galactopyranoside; b Data from (29); +, more than 90% of strains are positive; – 10% or less of strains are positive; ND, not determined; NR, not reported; d, 11–89% of strains are positive. *weak growth observed at 7% w/v NaCl.

Strain CC5C degrades a variety of hexoses, pentoses, L-methylpentoses, and sugar alcohols as summarized in (Supplementary Tables 1-3). Strain CC5C also has various enzyme activities including alkaline and acid phosphatase activity as well as C4 and C8 esterase activity (Supplementary Table 4). Additionally, strain CC5C degrades starch, casein, and β-glucan, a weak positive reaction on chitin, but does not degrade cellulose or xylan. Strain CC5C solubilizes phosphate on plates containing inorganic phosphate.

Supplementary Table 1 – API 20E Results for strain CC5C and *E. coli* (DSM 30083)

	β-Galactosidase	Arginine dihydrolase	Lysine decarboxylase	Ornithine decarboxylase	Citrate	H_2S Production	Urease	Tryptophan deaminase	Indole	Voges-Proskauer	Gelatin	Glucose	Mannitol	Inositol	Sorbitol	L-Rhamnose	Sucrose	Melibiose	Amygdalin	Arabinose	NO_3 to NO_2
Strain CC5C	+	(+)	+	+	-	-	-	-	+	-	+	+	+	+	+	+	(+)	(+)	+	+	+
E. coli (DSM 30083)	+	+	+	+	+	-	-	-	+	-	-	+	+	-	+	+	-	-	-	+	+

Supplementary Table 2 – API 20NE Results for strain CC5C and *E. coli* (DSM 30083)

	NO_3 Reductioin	Tryptophanase	Glucose	Arginine dihydrolase	Urease	Esculin	Gelatin	β-Galactosidase	Glucose	Arabinose	Mannose	Mannitol	*N*-Acetyl-Glucosamine	Maltose	Caprate	Adipate	Malate	Citrate	Phenylacetate
Strain CC5C	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+

Supplementary Table 3 – API 50CH results for strain CC5C *E. coli* (DSM 30083), and *E. marmotae* (DSM 28771, data from (19).

	Glycerol	Erythritol	D-Arabinose	L-Arabinose	D-Ribose	D-Xylose	L-Xylose	D-Adonitol	Me-β-D-Xylopyranoside	D-Galactose	D-Glucose	D-Fructose	D-Mannose	L-Sorbose	L-Rhamnose	Dulcitol	Inositol	D-Mannitol	D-Sorbitol	Me-α-D-Mannopyranoside	Me-α-D-Glucopyranoside	*N*-Acetylglucosamine	Amygdalin	Arbutin	Esculin	Salicin	D-Cellobiose	D-Maltose	D-Lactose	D-Melibiose	D-Saccharose	D-Trehalose	Inulin	D-Melezitose	D-Raffinose	Starch	Glycogen	Xylitol	Gentiobiose	Turanose	D-Lyxose	D-Tagatose	D-Fucose	L-Fucose	D-Arabitol	L-Arabitol	Potassium gluconate	Potassium 2-ketogluconate
Strain CC5C	(+)	-	+	+	+	+	-	-	-	+	+	+	+	-	+	-	-	+	+	-	+	+	-	-	+	-	-	+	+	+	+	+	-	-	+	(+)	-	-	-	-	-	-	+	-	-	+	-
E. coli (DSM 30083)	(+)	-	+	+	+	-	-	-	-	+	+	+	+	-	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	-	-	+	-	-	-	-	+	-	-	+	-
E. marmotae (DSM 28771)	+	N R	+	+		+	-	-	-	+	+	+	+	-	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	-	-	+	-	-	-	-	+	-	-	+	-

Supplementary Table 4 – API ZYM results for strain CC5C and *E. coli* (DSM 30083)

	Alkaline phosphatase	Esterase (C4)	Esterase Lipase (C8)	Lipase (C14)	Leucinearylamidase	Valinearylamidase	Cysteine arylamidase	Trypsin	Chymotrypsin	Acid phosphatase	Naphthol-AS-BI-phosphohydrolase	β-Galactosidase	α-Galactosidase	β-Glucuronidase	α-Glucosidase	β-Glucosidase	*N*-Acetyl-β-glucosaminidase	α-Mannosidase	α-Fucosidase
Strain CC5C	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
E. coli (DSM 30083)	+	(+)	-	-	+	-	-	-	-	+	+	+	-	-	+	-	-	-	-

3.3 FERMENTATION OF SINGLE SUBSTRATES

Single substrates and polymers present in various terrestrial and marine biomass were examined in anaerobic batch culture.

The end product formation patterns, from various substrates, were evaluated, and is presented for each individual substrate in Figure 3. 1,2-propanediol was only observed as a fermentation product when L-fucose and L-rhamnose were used as substrates.

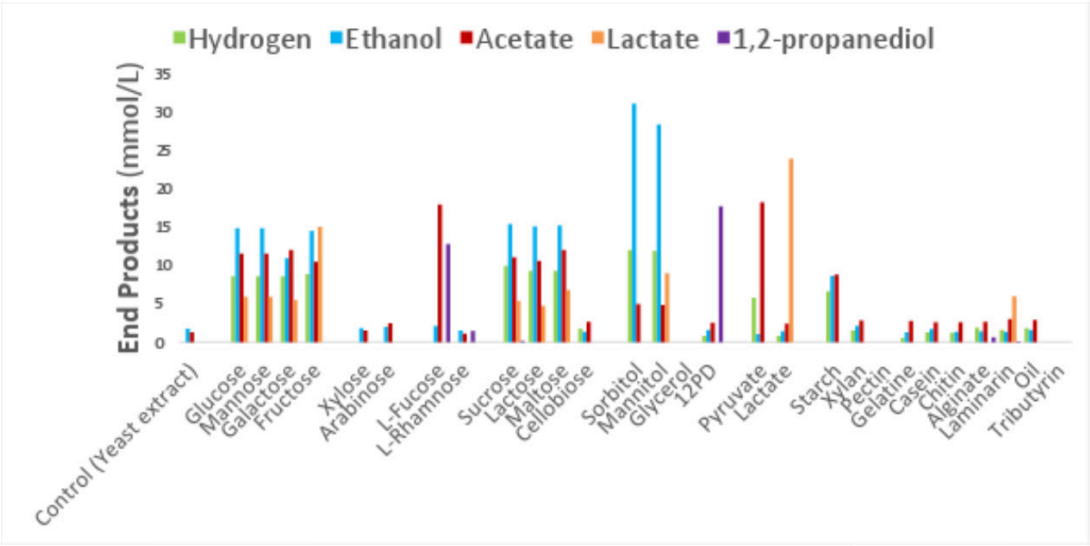

Figure 3 End products of selected substrates after fermentation by strain CC5C grown under anaerobic conditions; initial concentrations were 20 mM for monosaccharides and organic acids and polyalcohols, 10 mM for dissacharides, and 0.2% w/v for polymers. Values represent the average of triplicates; standard deviation is not shown.

Hexoses, pentoses, disaccharides except cellobiose, and starch gave a mixture of end products consisting of ethanol, acetate, lactate, and hydrogen. The sugar alcohols (mannitol and xylitol) yielded ethanol as a major end product with traces of acetate, lactate, and hydrogen. Strain CC5C fermented methylpentoses (L-fucose and L-rhamnose) and yielded 1,2-propanediol as the dominant end product with acetate and lactate present in lower concentrations. Laminarin, a β-1,3 glycan, yielded acetate and lactate above controls while other polymers, lactate, and *rac*-1,2-PD were not degraded.

3.4 EFFECT OF INITIAL SUBSTRATE CONCENTRATION AND OXYGEN ON 1,2-PROPANEDIOL YIELDS

To investigate the production of 1,2-propanediol by strain CC5C, the strain was evaluated at different initial substrate concentrations up to 60 mM on D-glucose, L-fucose, and L-rhamnose under both aerobic and anaerobic conditions.

End products were evaluated after 5 days of incubation as shown in Figure 4 for both aerobic (4A) and anaerobic conditions (4B).

Substrate inhibition was observed on all substrates at concentrations greater than 20 mM as evidenced by the decreased substrate utilization and levelling off of end product formation.

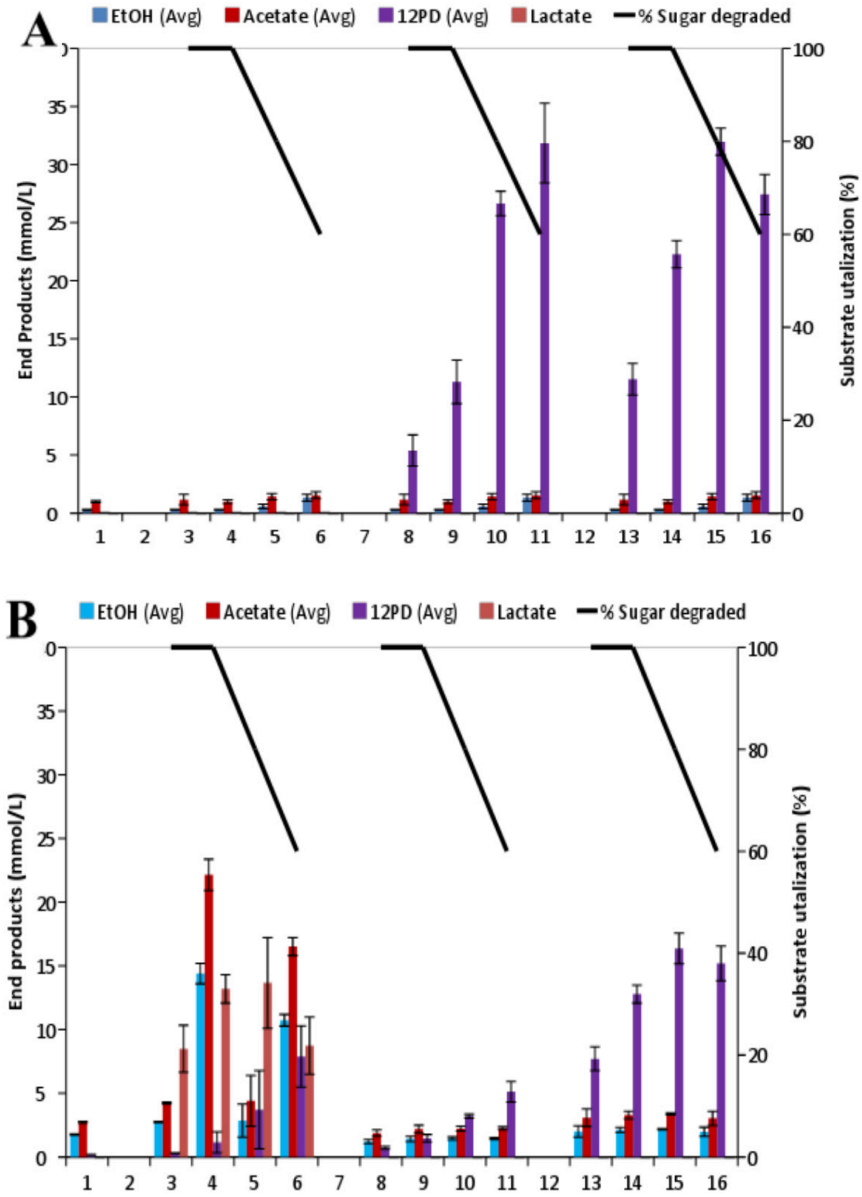

Figure 4 End product formation of strain CC5C on glucose, L-rhamnose, and L-fucose under aerobic (A) and anaerobic (B) conditions. Values represent the average of triplicates with standard deviation shown as error bars.

3.5 KINETIC EXPERIMENTS

Kinetic experiments were performed aerobically on 20 mM of D-glucose, L-rhamnose, L-fucose, a mixture of D-glucose and L-rhamnose (20 mM each), and 20 mM of *rac*-1,2-propanediol as shown in Figure 5A-D, as well as a control containing only yeast extract. The fermentation characteristics for the production of 1,2-PD are summarized in Table 2.

Table 2 – Fermentation Characteristics of 1,2 propanediol production on selected substrates

Substrate (20mM)	Maximum 1,2-PD Yield (% of Theoretical)	Maximum 1,2-PD Production Rate (mmol/hr)
D-Glucose	9.93	0.99
L-Rhamnose	96.89	3.41
Glucose + Rhamnose	75.84	2.37
Rac-1,2-PD	69.90	-1.86

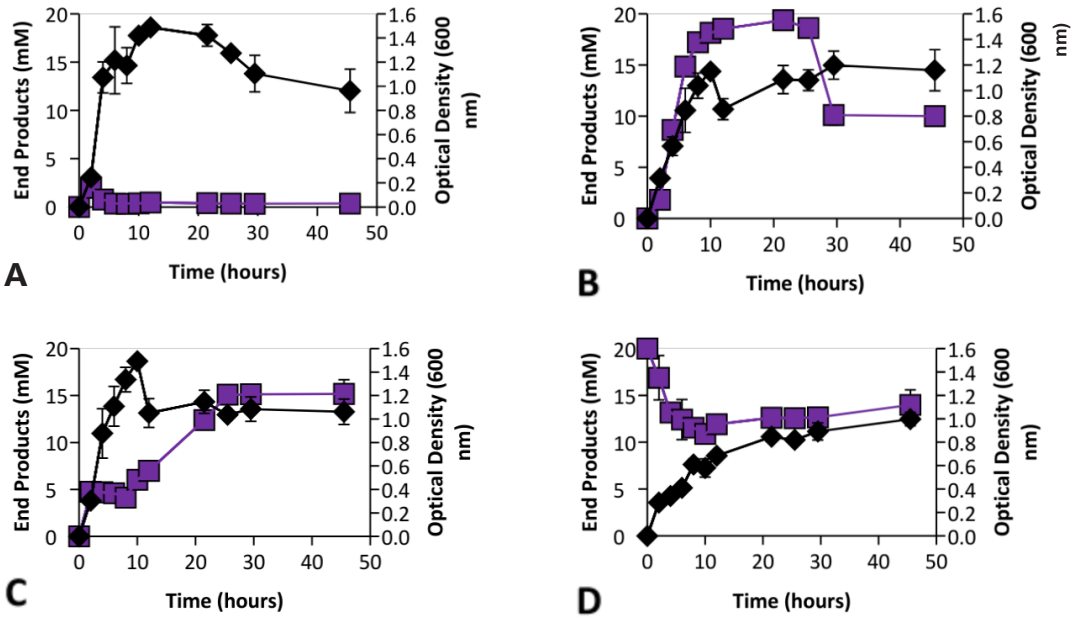

Figure 5 – Utilization of selected substrates over time by strain CC5C. Values represent the average of triplicates with standard deviation presented as error bars. A: 20 mM Glucose, B: 20 mM L-Rhamnose, C: 20 mM Glucose and 20 mM L-rhamnose, D: 20 mM *rac*-1,2-propanediol; ■ – 1,2-propanediol, ♦ - optical density (600 nm).

Strain CC5C rapidly catabolizes glucose and rhamnose and produces 1,2-PD at a rate of 3.41 mmol/h on L-rhamnose and 2.37 mmol/h on a mixture of glucose and rhamnose with up to 96% on L-rhamnose. Given a solution of *rac*-1,2-PD, strain CC5C degrades approximately half of the initial 1,2-PD concentration at a rate of 1.86 mmol/h.

DISCUSSION

Strain CC5C is very closely related to a number of organisms within the genera of *Escherichia* and *Shigella*, the latter of which are widely regarded as likely being representatives of *E. coli* clones (25). Other techniques, such as DNA-DNA hybridization are required to determine conclusively if strain CC5C represent a novel species given the high degree of similarity among species within these genera.

Strain CC5C's T_{opt} matches the temperature of the geothermal pool (40°C) from which it was isolated from. Similarly, *E. marmatoae* can grow at 25–37°C, and with 0–6 % NaCl with optimum growth conditions are 37°C and 1 % NaCl. Interestingly, enteric bacteria are not typically associated with a highly-saline marine environment or geothermal feature as *Escherichia* species are typically associated with the digestive tract of mammals and, less commonly, other animals. However, there is at least one example of an organism within family *Enterobacteriaceae* being found in intertidal hot springs, that being the moderately thermophilic halophile *Alterococcus agarolyticus* which was isolated in Taiwan (32). Their secondary presence in other environments is often the result of fecal contamination (32), thus the presence of this organism could be the result of nesting birds near the sampling location observed at the time that the sample was taken. The high conductivity of the sample taken from the pool, which is equivalent to approximately 3.1% sodium chloride is less saline than seawater. As members of the family *Enterobacteriaceae* are typically not halophilic (Brenner, 2005), it is somewhat surprising that strain CC5C persists in this environment. Strain CC5C may be thriving in this environment due to the influx of fresh geothermal water into the intertidal pool from the geothermal system and due to the abundance of marine biomass in the pool such as protein and carbohydrates from macro algae. Metagenomic studies of these intertidal pools over the course of multiple seasons are needed to resolve whether strains such as CC5C are stable members of the geothermal intertidal community or not.

Strain CC5C is phenotypically similar to *E. coli* and other closely related species with several notable differences in substrate utilization and growth conditions as compared to related species; the majority of the biochemical tests reveal traits similar to other enteric bacteria that are facultative anaerobes within the family of *Enterobacteriaceae*. From a purely taxonomic standpoint, a lack of lysine decarboxylation is a defining feature of the genus *Shigella*, and therefore it is suggested that CC5C is a member of the genus *Escherichia*. Strain CC5C has a broader temperature range than both *E. marmatoa* (reported as only growing between 25 and 37°C) and *E. coli* while demonstrating higher specific growth rates at 40 and 45°C as compared to *E. coli* (DSM

30083) under equivalent conditions. While strain CC5C is not thermophilic, it does seem better adapted to growth at elevated temperatures than the reference strain used (*E. coli* DSM 30083). Additionally, CC5C′s growth rates at elevated salt concentrations are higher than the type strain of *E. coli*. This may suggest that CC5C has adapted to its environmental niche (40°C or more with regular saline intrusion).

CC5C has a broader substrate spectra than closely related species as it degrades hexoses, pentoses, methylpentoses, sugar alcohols, and disaccharides as well as some polysaccharides. Four substrates stand out at differentiating strain CC5C from its nearest phylogenetic neighbours, namely the utilization of starch, sucrose, dulcitol, and possibly cellobiose. Strain CC5C degrades starch as evidenced by positive tests on both the API 50CH test strips, starch plate assay, as well as the appearance of organic acids under fermentative conditions suggesting the strain has the required amylase activity and potentially an import mechanism for maltose, which is supported by growth on maltose, or higher order oligosaccharides. This stands in contrast to other organisms within the genus of *Escherichia*, which cannot degrade starch but can utilize maltose. Remarkably, strain CC5C utilizes cellobiose and sucrose, which is uncharacteristic for *E. coli* and other closely related *Enteroacteriaceae*. A follow-up experiment examining the fermentation of cellobiose found that no end products were detected suggesting that the result of the API 50CH test strip was a false positive or utilization of cellobiose is limited to aerobic conditions. The utilization of dulcitol, the sugar alcohol formed from the reduction of galactose, is a somewhat unusual feature. As can be seen in Table 1, CC5C′s substrate spectra is more similar to *E. coli* than *E. marmatoa*, its nearest phylogenetic neighbour based on analysis of the 16S gene but also possesses features somewhat similar to *E. coli* with some of the aforementioned differentiating characteristics. The number of divergent features may suggest that CC5C is a novel species within the genus.

As the environment from which CC5C was isolated is rich with macro algae species with high protein fractions as well as polysaccharides containing methylpentoses and other sugars not common within the terrestrial biosphere, the strain will likely thrive in this substrate-rich niche. The strain also has acid and alkaline phosphatase activities suggesting that CC5C might serve a role mobilizing phosphate in the environment. CC5C also degrades casein and gelatin but lacks trypsin and chymotrypsin activities suggesting other protease chemistries are present. The strain showed esterase activity but not lipase activity, which is surprising since this bacterium was enriched on rapeseed oil; this might suggest that CC5C was utilizing the yeast extract present in the medium. It is worth noting that CC5C possessed at C8 esterase activity, unlike *E. coli* (DSM 30083). CC5C also showed phosphatase activity which may help the strain in scavenging phosphate from inorganic or polyphosphates in the environment to support growth.

To further examine the end products produced by CC5C, the fermentation of selected substrates was examined. Under anaerobic conditions, the dominant end products are a mixture of ethanol, acetate, and hydrogen on most substrates examined, with the exception of L-fucose and L-rhamnose, which yielded 1,2-PD. The observed substrate spectrum and end product formation patterns observed for strain CC5C is quite similar to other members of the family of

Enterobacteriaceae, with members such as *E. coli* being well-established mixed acid producers as well as producers of 1,2-PD in the presence of methylpentoses.

To further investigate the production of 1,2-PD from CC5C, the strain was cultivated in the presence of D-glucose as well as the methylpentoses L-fucose and L-rhamnose at initial concentrations ranging from 10 mM to 60 mM. Strain CC5C was clearly inhibited by high initial substrate concentrations of all three monosaccharides examined under aerobic and strictly anaerobic conditions. 1,2-PD was the primary end product when CC5C was grown on L-rhamnose and L-fucose under both aerobic and anaerobic conditions. Interestingly, very little 1,2-PD was produced from L-rhamnose under anaerobic conditions at all concentrations. Under aerobic conditions, 1,2-PD yields were much higher although inhibition phenomena were apparent at higher substrate concentrations. This stark shift in end product formation could implicate the importance of oxygen in the ability of the strain to produce 1,2-PD. It is known that during the production of 2,3-butanediol by organisms such as *Paenibacillus polymyxa*, that the oxygen concentration is of critical importance (22). Furthermore, studies in the 1980s examined changes in the regulation, expression and activity of propanediol oxidoreductase by *E. coli* under specific oxygen conditions (5, 7). It is possible that reducing potential from rhamnose metabolism is being shifted to other end products or that highly reduced conditions interfere with enzymes necessary for rhamnose catabolism that are specific to rhamnose regulation but do not affect the enzymes involved in fucose catabolism. The presence and organization of the rhamnose and fucose operons, if present, should be investigated via whole genome sequencing or specific assays to confirm the presence and functionality of specific gene products. Analysis of CC5C's transcriptome, as well as the activities of specific enzymes, such as propanediol oxidoreductase as a function of oxygen concentration, should also be considered in future work.

At high glucose concentrations, the amount of 1,2-PD increased to 7.9 mM at an initial glucose concentration of 60 mM as compared to only 0.3 mM of 1,2-PD at an initial glucose concentration of 10 mM. This may be due to the rapid metabolism of glucose to glycolysis intermediates such as dihydroxyacetone phosphate (DHAP), while further metabolism to other end products was inhibited. To proceed through glycolysis, DHAP must be converted to glyceraldehyde-3-phosphate (G3P), which serves as a metabolic bottleneck resulting in carbon flowing into the methyglyoxal bypass. This pathway is well known to produce *(R)*-1,2-propanediol in *E. coli* and yeasts and could be active in *Thermoanaerobacterium saccharolyticum* HG-8 (3). Phosphate limitation could also be a factor at higher initial substrate concentrations as phosphate limiting conditions is known to influence the flow of carbon into the methylglyoxal pathway as has been demonstrated with *Clostridium sphenoides* (31). There have been many recent efforts to engineer *(R)*-1,2-PD producing pathways into *E. coli* to improve yields although efforts to improve *(S)*-1,2-PD production have been largely ignored.

A kinetic study of 1,2-PD production by strain CC5C reveals that the methylpentoses are rapidly consumed while 1,2-PD production lags. This suggests that the metabolism of intermediates involved in methylpentose catabolism is slower than that of the glycolytic intermediates of cells grown on glucose.

CC5C partially degraded *rac*-1,2-PD to roughly 50% of its initial concentration. This suggests that the strain may only degrade one enantiomer of 1,2-PD. As the 1,2-PD concentrations were stable in the experiments using L-fucose and L-rhamnose as a carbon source CC5C likely produces the S enantiomer. It is likely that CC5C can only degrade the R enantiomer or degrades one enantiomer at a faster rate. This may have potential biotechnological applications in the production of enantiomerically pure 1,2-PD from inexpensive commercially available racemic 1,2-PD as has been done with strains of Baker's yeast (16). Further work is needed to confirm the enantiomeric selectivity of 1,2-PD degradation by CC5C.

The high yields of 1,2-PD observed on L-fucose are promising although L-fucose is an expensive substrate. Thus, inexpensive sources of L-fucose should be examined, potentially including hydrolysates of components found in brown macro algae. Further work to evaluate the ubiquity of methylpentose metabolism resulting in 1,2-PD production by members of the genus requires further examination. Additionally, bioprospecting efforts to find 1,2-PD producing strains adapted in situ to more extreme environments as well as possessing expanded substrate spectra would be of potential interest to the industry.

Further work is needed to investigate the influence of culture parameters, such as the partial pressure of oxygen and hydrogen, effect of pH and temperature, as well as the effect of phosphate limitation on the production of 1,2-PD on specific substrates. The molecular biology involved in the changes in 1,2-PD production from L-rhamnose under aerobic versus strictly anaerobic conditions warrants further study. The response of CC5C to inhibitory compounds such as 5-hydroxylmethylfurfuraldhyde and 2-furfuraldehyde, and production of 1,2-PD from biomass, containing methylpentoses should be investigated. Due to the observed substrate inhibition, other fermentations modes such as fed batch or continuous culture should be investigated. Also, the inhibitory effects of end products, such as acetate, lactate, and 1,2-PD, should be investigated to better understand whether the observed inhibition is solely to the initial concentration of the substrate or another type of inhibition.

CONCLUSIONS

Strain CC5C is a Gram-negative, facultative anaerobe isolated from a geothermally-heated intertidal pool in Husavik, Iceland, and is closely related to *Escherichia marmotae* (99.13%) and *Shigella dysenteriae*(99.13%). The strain had optimum growth conditions of 40°C, pH 7.5 and 0.5% NaCl with a maximum specific growth rate of 1.10 h^{-1}. The strain has a broad substrate spectrum and grew on a number of substrates (dulcitol, cellobiose, starch, and sucrose) unlike a number of closely related species potentially suggesting that CC5C is a novel isolate. Strain CC5C is also a potent 1,2-propaendiol producing organisms fermenting methylpentoses to 1,2-PD in good yield (>95% of theoretical) although some 1,2-PD was observed on glucose under high initial substrate concentrations (aerobic versus anaerobic). Strain CC5C had a maximum production rate of 3.41 mmol/L/h from L-rhamnose under aerobic conditions.

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